Inflammation and matrix remodeling during repair of ventilator-induced lung injury

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Departments of 1Biología Funcional, 2Cirugía y Especialidades Médico-quirúrgicas, and 3Medicina, Universidad de Oviedo, Instituto Universitario Oncologico del Principado de Asturias, Departments of 4Medicina Intensiva, 5Anatomía Patológica, and 6Immunología, Hospital Universitario Central de Asturias, and 7CIBER-Enfermedades Respiratorias, Oviedo, Spain

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González-López A, Astudillo A, García-Prieto E, Fernández-García MS, López-Vázquez A, Batalla-Solís E, Taboada F, Fueyo A, Albaiceta GM. Inflammation and matrix remodeling during repair of ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol 301: L500–L509, 2011. First published July 8, 2011; doi:10.1152/ajplung.00010.2011.—High-pressure ventilation triggers different inflammatory and matrix remodeling responses within the lung. Although some of them may cause injury, the involvement of these mediators in repair is largely unknown. To identify mechanisms of repair after ventilator-induced lung injury (VILI), mice were randomly assigned to baseline conditions (no ventilation), injury [90 min of high-pressure ventilation without positive end-expiratory pressure (PEEP)], repair (injury followed by 4 h of low-pressure ventilation with PEEP), and ventilated controls (low-pressure ventilation with PEEP for 90 and 330 min). Histological injury and lung permeability increased during repair, but were partially reverted in the repair group. This was accompanied by a proinflammatory response, together with increases in TNF-α and IFN-γ, which returned to baseline during repair, and a decrease in IL-10. However, macrophage inflammatory protein-2 (MIP-2) and matrix metalloproteinases (MMP)-2 and -9 increased after injury and persisted in being elevated during repair. Mortality in the repair phase was 50%. Survivors showed increased cell proliferation, lower levels of collagen, and higher levels of MMP-2 and MMP-2. Pan-MMP or specific MMP-2 inhibition (but not MMP-2, TNF-α, or IL-4 inhibition) delayed epithelial repair in an in vitro wound model using murine or human alveolar cells cultured in the presence of bronchoalveolar lavage fluid from mice during the repair phase or from patients with acute respiratory distress syndrome, respectively. Similarly, MMP inhibition with doxycycline impaired lung repair after VILI in vivo. In conclusion, VILI can be reverted by normalizing ventilation pressures. An adequate inflammatory response and extracellular matrix remodeling are essential for recovery. MMP-2 could play a key role in epithelial repair after VILI and acute respiratory distress syndrome.

THE OUTCOME OF PATIENTS WITH THE ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) IS RELATED TO THE REDUCTION OF VENTILATOR-ASSOCIATED LUNG INJURY. RESEARCH ON ITS EXPERIMENTAL COUNTERPART, CALLED VENTILATOR-INDUCED LUNG INJURY (VILI), HAS IDENTIFIED A VARIETY OF MECHANISMS OF LUNG DAMAGE. THE INITIAL INSULT TO THE LUNGS IS PHYSICAL IN NATURE (12); THUS THE MAIN THERAPEUTIC STRATEGY IS THE OPTIMIZATION OF MECHANICAL VENTILATION. THIS APPROACH HAS BEEN SHOWN TO BE EFFECTIVE IN REDUCING MORTALITY (5). HOWEVER, NO SINGLE VENTILATORY SETTINGS EXIST THAT CAN MINIMIZE ALL OF THE PATHOGENETIC MECHANISMS OF INJURY [I.E., INCREASING POSITIVE END-EXPIRATORY PRESSURE (PEEP) CAN DECREASE CYCLIC CHANGES IN AERATION, BUT INCREASES THE RISK OF ALVEOLAR OVERSTRETCHING (4)]. THE MECHANICAL STIMULUS INDUCES A SECONDARY BIOCHEMICAL RESPONSE IN WHICH INFLAMMATION (37) AND EXTRACELLULAR MATRIX REMODELING (32) ARE KEY PROCESSES. ALTHOUGH DIFFERENT PHARMACOLOGICAL APPROACHES HAVE DEMONSTRATED A REDUCTION IN VILI (2, 20, 29, 31), THEY HAVE NOT BEEN TRANSLATED INTO THE CLINICAL PRACTICE AND ALSO POSE A RISK OF ADVERSE EVENTS. FOR EXAMPLE, MODULATION OF THE IMMUNE RESPONSE CAN LEAD TO AN INCREASED INCIDENCE OF INFECTION.

THE RELEVANCE OF LUNG REPAIR IN THIS SCENARIO IS LARGELY UNKNOWN (11). PIONEERING WORK BY NIN ET AL. (28) HAS DEMONSTRATED THAT VILI CAN BE REVERTED AFTER REESTABLISHING SPONTANEOUS BREATHING. FURTHERMORE, SOME OF THE MEDIATORS RELEASED DURING LUNG INJURY, SUCH AS IL-1 (33) AND MATRIX METALLOPROTEINASE (MMP)-9 (30), PROMOTE EPITHELIAL REPAIR AND MAY HAVE PROGNOSTIC AND THERAPEUTIC IMPLICATIONS IN ARDS PATIENTS. INTERFERENCE WITH THESE MOLECULES CAN FURTHER INCREASE LUNG DAMAGE (3). CONVERSELY, THERAPIES AIMED TO PROMOTE LUNG REPAIR MAY BE AN ALTERNATIVE IN PATIENTS SUBMITTED TO MECHANICAL VENTILATION.

THE OBJECTIVE OF THIS STUDY IS TO DESCRIBE A MODEL OF REPAIR AFTER VILI, FOCUSING ON INFLAMMATORY MEDIATORS AND EXTRACELLULAR MATRIX REMODELING. ADDITIONALLY, ASSESSMENT OF THE EFFECTS OF THESE MEDIATORS ON EPITHELIAL LUNG EPITHELIAL WOUND CLOSURE COULD HELP TO IDENTIFY THOSE INVOLVED IN LUNG REPAIR.

MATERIALS AND METHODS

Animals. Eight- to twelve-week-old CD1 mice were used in all experiments. Mice were kept under specific pathogen-free conditions, with free access to food and water, and exposed to 12:12-h light-dark cycles. The protocol was approved by the Committee on Animal Experimentation of the Universidad de Oviedo, Spain. The use of human samples was authorized by the Regional Ethics Committee, and informed consent was obtained from patients’ next of kin.

Experimental protocol. Mice were anesthetized with a mixture of ketamin and xylazin administered intraperitoneally and placed on a heating pad. A tracheostomy was performed; a 20G catheter was inserted in the trachea and tightened to avoid air leaks. The animals were then ventilated (Evita 2 Dura with Neoflow, Drager, Germany). A 0.2-ml bolus of Ringer lactate was injected intraperitoneally at the onset of ventilation and repeated every 2 h. Anesthesia was maintained by administration of ketamin and xylazin, as needed.

Animals were ventilated in pressure-controlled mode using one of two ventilatory settings: high-pressure ventilation, peak inspiratory...
pressure (PIP) 25 cmH2O, PEEP 0 cmH2O, respiratory rate 50 breaths/min; and low-pressure ventilation, PIP 15 cmH2O, PEEP 2 cmH2O, respiratory rate 100 breaths/min. Fraction of inspired O2 was 50%, and inspiratory-expiratory ratio was 1:1. The combination of these two ventilatory strategies with different ventilatory times led to the creation of five experimental groups (Table 1). Specifically, the group ventilated with high pressures for 90 min, followed by 240 min of low-pressure ventilation, was aimed to study repair after VILI. The mortality in this group was 50%, and all of the animals, irrespective of the time of ventilation, were included in the subsequent cellular, biochemical, and histological analysis. Animals were randomly assigned to each experimental group. The main objective was to obtain at least 16 mice surviving the repair phase, and mice were added in pairs to the “repair” and another group (randomly chosen) in parallel until reaching that objective, resulting in different sample sizes (Table 1). Additional mice (n = 12) were ventilated using high pressures until death. This group was used only for the survival analysis.

After ventilation or immediately after death, a laparotomy was performed, and mice were killed by exsanguination, the chest was opened, the heart-lung block was removed, and the right bronchus was ligated. The right upper lobe was weighed, dried in an oven (50°C for 72 h), and weighted again to calculate the wet-to-dry weight ratio. The remaining right lung was homogenized in a lysis buffer containing 20 mM Tris, 300 mM sucrose, 1% Triton X-100, and a protease inhibitor cocktail without EDTA (Complete, Roche, Germany), centrifuged (13,000 rpm for 15 min), and stored at −80°C for further studies. The protein content of the homogenate was quantified (BCA protein assay, Pierce, Rockford, IL).

Histological study. The left lung was fixed intratracheally with 300 μl of formaldehyde, tied, and immersed in the same fixative for at least 24 h. After fixation, the left lung was embedded in paraffin and processed for a standard hematoxylin-eosin staining. One pathologist blinded to the experimental conditions scored three slides of the left lung according to the following scale: 0, normal lung; 1, septal congestion; 2, epithelial thickening; 3, septal inflammatory infiltrates; 4, alveolar hemorrhage and/or hyaline membranes; 5, massive disruption of lung architecture. To study cell proliferation during the repair phase, the number of nuclei in the alveolar walls was counted in three randomly chosen high-power fields (∗×400). Additional lungs from animals during the injury and repair phases were processed for electron microscopy studies. Lung tissues were fixed in 3% glutaraldehyde and osmium tetroxyde, and semithin sections were stained with toluidine blue. After selection of regions of interest, ultrathin sections were collected, stained with uranyl acetate and lead citrate, and observed in a JEOL 1011 transmission electron microscope.

Immunohistochemistry studies were done in paraffin-embedded sections using antibodies against myeloperoxidase (MPO, Thermo Scientific), MMP-2 (Abcam), MMP-9 (Santa Cruz Biotechnology SC-6840), and Ki-67 (Abcam). The number of MPO-positive cells was counted in three randomly chosen ×200 fields and averaged. The percentage of Ki-67 positive nuclei was computed as an index of cell proliferation.

Bronchoalveolar lavage. In additional mice, submitted to the same ventilatory strategies described above, a lavage was performed via the tracheal catheter using three aliquots (0.7 ml) of saline. Two hundred microliters of the recovered bronchoalveolar lavage fluid (BALF) were used to measure cell count and population in a hemocytometer; the remaining volume was centrifugated (2,000 rpm for 15 min) and stored at −80°C for further analysis. The protein content of BALF fluids was quantified (BCA protein assay, Pierce). No other samples were harvested from these animals.

Cytokine/chemokine quantification. IFN-γ, IL-10, IL-4, MIP-2, and LPS-induced CXC chemokine (LIX) were measured in lung homogenates using a multiplex assay (Milliplex kit, Millipore), according to the manufacturer’s instructions and a LumineX 100 system. TNF-α was quantified using a commercial ELISA kit (mouse TNF-α ELISA kit, eBioscience).

Collagen measurement. Soluble lung collagen was measured in lung homogenates using the Sircol assay (Biocolor), following the manufacturer’s instructions.

Quantification of MMP-2 and MMP-9. MMP-2 and MMP-9 activity was measured in lung homogenates and BALF by gelatin zymography. The volume of lung homogenate corresponding to 15 μg of protein or 6 μl of BALF was loaded in an 8% SDS-polyacrylamide gel containing 0.2% gelatine and electrophoresed. Afterwards, gels were washed twice in 2.5% Triton X-100 for 15 min, then washed again with deionized water until the complete removal of Triton X-100, and incubated overnight at 37°C in a buffer containing 20 mM Tris-HCl, 5 mM CaCl2, pH = 7.4. Then gels were stained using Commassie blue, destained with a mixture of acetic acid and methanol, and scanned. Intensity of the gelatinolytic bands was quantified (in arbitrary density units) using ImageJ software (National Institutes of Health). All gels contained at least one sample of each experimental group to overcome differences among gels.

Wound healing in cultured alveolar cells. The effect of different mediators on epithelial repair was studied using cell cultures. Murine alveolar epithelial cells (MLE-12, ATCC) were cultured in 24-well plates in DMEM/Ham’s F-12 supplemented with 2% fetal bovine serum and glutamine. After confluence, cells were wounded with a pipette tip and washed with PBS. Cells were then cultured using DMEM/Ham’s F-12 medium supplemented with 10% BALF from ventilated mice after injury or repair (filtered using an Acrodisc 0.2-μm filter, Pall Life Sciences). Cells cultured in medium without BALF were used as controls. To assess the effect of mediators on wound healing, blocking antibodies against TNF-α (eBioscience), IL-4 (eBioscience), MIP-2 (AbD Serotec), and MMP-2 (Abcam) were added to the medium in parallel experiments. Additionally, all cells were cultured in the presence of doxycycline (25 μM) to test the effects of nonspecific pan-MMP inhibition (16). Cultures were photographed using an Olympus BH-2 microscope and an Olympus C-5060 camera. Images were obtained immediately after wounding and 3, 6, 9, and 12 h later. The area not covered by cells was measured using the ImageJ software, and the percentage of the initial wound area covered by cells over time was computed.

To study the contribution of MMP-2 to wound closure in patients with ARDS, a similar assay was performed using human alveolar cells (A549). First, BALF from patients meeting ARDS criteria and who had had less than 5 days of mechanical ventilation, who also underwent a diagnostic bronchoscopy, was filtered through a sterile gauze and centrifuged at 3,000 rpm for 15 min; the supernatant was stored at −80°C. Four samples were finally collected. Informed consent for the use of these BALF samples was obtained from each patient’s next of kin. A549 cells were cultured in DMEM supplemented with 10% FBS until confluence, wounded as previously described, washed and cultured using DMEM with 10% BALF from the patients meeting the described criteria, and filtered (Acrodisc 0.2-μm filter, Pall Life Sciences). Again, cells grown in medium without BALF were used as controls. In duplicate cultures, an anti-MMP-2 blocking antibody was added to the medium. As doubling time is longer in A549 cells under these conditions, cultures were photographed every 24 h for 4 days. Wound area was measured as previously described.

Table 1. Composition of the experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Time of Ventilation, min</th>
<th>High pressure</th>
<th>Low pressure</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>10/11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Injury</td>
<td>12/15</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>Repair</td>
<td>17/15</td>
<td>90</td>
<td>240</td>
</tr>
<tr>
<td>Control 90 min</td>
<td>11/9</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>Control 330 min</td>
<td>7/10</td>
<td>0</td>
<td>330</td>
</tr>
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</table>

BALF, bronchoalveolar lavage fluid.
Effects of MMP inhibition on lung repair in vivo. The effects of MMP inhibition in vivo were studied using additional mice. Due to the lack of specific MMP-2 inhibitor available for in vivo use, doxycycline was administered as a pan-MMP blocker. The animals were submitted to 90 min of injurious ventilation. Then a dose of 50 mg/kg of doxycycline or vehicle was administered intraperitoneally, and the ventilatory settings were switched to low pressures to allow the lungs to repair. Vehicle-treated mice were studied after the same ventilatory time than doxycycline-treated animals. The lungs were harvested, and histological sections scored as previously described.

Statistical analysis. Data are expressed as means ± SE. Results were compared using a one-way ANOVA. Post hoc tests were done when appropriate using the Dunnett test to compare against baseline or injury groups. Survival was studied using Kaplan-Meier curves and the log-rank test. Differences between the animals that did or did not survive through the repair phase, or between doxycycline- and vehicle-treated mice, were compared using a T-test. Results from wound healing assays were compared using a repeated-measurements ANOVA. A P value <0.05 was considered significant. All the calculations were done using SPSS 17.0 software (SPSS).

RESULTS

One hundred twenty-nine animals were included in the study. The composition of each experimental group is presented in Table 1.

Reversibility of lung injury. VILI results in structural damage of lung parenchyma and increased lung edema and permeability. After 90 min of high-pressure ventilation, mice showed a 6-fold increase in the injury score (Fig. 1A), a 62% increase in the wet-to-dry weight ratio (Fig. 1B), and a 10-fold increase in BALF protein concentration (Fig. 1C) compared with baseline (P < 0.001 in all post hoc tests). All of these changes were partially reverted during repair, with significant decreases in the histological score, the wet-to-dry weight ratio, and the BALF protein concentration. Representative optical and electron microscopy images are presented in Fig. 1, D and E. Increases in epithelial cell size, septal thickness, and deposition of interstitial fibers were observed during injury and decreased during the repair phase.

In addition, results from mice ventilated with low pressures for 330 min showed that prolonged ventilation with lower pressures can also induce a mild lung injury.

Collectively, these results demonstrate that lung injury caused by high-pressure ventilation can be partially reversed after 4 h of ventilation with low pressures and PEEP.

Proinflammatory response to high-pressure ventilation can be rapidly reverted. The inflammatory response to mechanical ventilation was studied by quantification of cells in BALF and immune mediators in lung tissue. There were no significant differences in leukocyte counts in BALF (P = 0.2 in the ANOVA test, Fig. 2A). However, a change in cell populations was observed (Fig. 2B). The number of macrophages decreased...
significantly after 90 min of injurious ventilation ($P < 0.01$ in all post hoc tests) and was similar to baseline values in all of the other groups. There were no significant changes in the absolute count of neutrophils or lymphocytes in BALF. However, immunohistochemical studies (Fig. 4) showed an increase in neutrophils, from $30 \pm 2$ cells/high power field at baseline to $88 \pm 8$ cells/high power field after injury and $88 \pm 9$ cells/high power field during repair, suggesting that these cells were firmly attached to the epithelium and were not recovered in BALF.

To characterize the humoral inflammatory response in the different ventilatory groups, we determined the concentration of Th1 cytokines (TNF-$\alpha$, IFN-$\gamma$), Th2 cytokines (IL-4, IL-10), and chemokines (MIP-2 and LIX) in lung homogenates (Fig. 2, C–H). Injurious ventilation significantly increased levels of TNF-$\alpha$ compared with other groups (Fig. 2C, $P < 0.05$ in ANOVA and all post hoc tests). Mice submitted to further ventilation with low pressures for 4 h (repair) showed TNF-$\alpha$ levels similar to those of nonventilated controls ($P = 0.998$ in post hoc test). There were no significant differences between mice in baseline conditions (nonventilated) and after low-pressure ventilation. We observed also a trend toward higher levels of IFN-$\gamma$ in mice ventilated with high pressures ($P = 0.098$ in ANOVA). Again, values during the repair phase were similar to baseline.

Regarding Th2 cytokines, there were no significant changes in IL-4 (Fig. 2E, $P = 0.2$ in ANOVA). However, IL-10 (Fig. 2F) decreased in mice submitted to high-pressure ventilation ($P < 0.05$ in ANOVA, $P = 0.001$ in post hoc test compared with baseline). This decrease was also significant in the repair phase ($P = 0.001$) and after prolonged low-pressure ventilation ($P = 0.008$).

The increase in neutrophils suggested that chemokines may play a relevant role in this kind of injury. MIP-2 significantly increased after 90 min of high-pressure ventilation (Fig. 2G, $P = 0.004$ in ANOVA, $P < 0.03$ in post hoc tests against baseline and ventilated control groups). Contrary to what we observed with cytokines, the decrease present during repair

Fig. 2. Inflammatory response to ventilation. There were no significant differences in total cell count (A), but cell types differed among the different groups (B). Additionally, different mediators were measured in lung tissue homogenates. There were increases in proinflammatory cytokines, such as TNF-$\alpha$ (C) and IFN-$\gamma$ ($P = 0.098$; D), and a decrease in the anti-inflammatory cytokine IL-10 (F). G: there was also a marked increase in the chemokine macrophage inflammatory protein-2 (MIP-2) that persisted throughout the repair phase. No significant differences in IL-4 (E) and LPS-induced CXC chemokine (LIX; H) were observed. Values are means $\pm$ SE. $^# P < 0.05$ in post hoc test compared with baseline. $^† P < 0.05$ in post hoc test compared with injury.
was not significant (P = 0.46 in post hoc test against injury). There were no differences among groups in LIX levels (Fig. 2H).

Collectively, these differences show that high-pressure ventilation induces the release of proinflammatory cytokines and a decrease in anti-inflammatory cytokines; also, chemokine increase is more prolonged.

**MMPs-2 and -9 increase during tissue repair.** The extracellular matrix plays a key role in lung injury and repair. We evaluated collagen and MMPs-2 and -9 in lung homogenates. There were no differences in collagen content among groups (P = 0.259 in ANOVA, data not shown).

There were significant changes in MMP-9 and MMP-2 (Fig. 3, P < 0.01 in ANOVA in both cases). MMP-9 increased in lung tissue from all ventilated groups (P < 0.01 in post hoc tests when compared against baseline), and in BALF from animals during the injury and repair phase, as well as after prolonged low-pressure ventilation. MMP-2 followed a similar pattern. MMP-2 and -9 were detected by immunohistochemistry in parafin-embedded sections. MMP-2 was expressed mainly in epithelial and interstitial cells, although some intra-alveolar inflammatory cells were also positive. However, MMP-9 expression was restricted to inflammatory cells. Figure 4 shows representative sections (×200) of baseline, injury, and repair groups (survivors and nonsurvivors). The difference in MMP-2 expression between survivors and nonsurvivors was also confirmed in this immunohistochemical study.

The increased levels of MMPs during repair, despite a decrease in lung damage indexes, suggest that these molecules may have a role in tissue repair.

**Survival.** All of the mice subjected to low-pressure ventilation survived the 330 min of the experiment. In contrast, ventilation with high pressures for >90 min led to 100% mortality. Animals assigned to the repair group had a mortality of 50% (8 animals assigned to tissue sampling and 8 assigned to BALF survived). Survival curves are shown in Fig. 5A. The difference between curves was significant, as assessed by the log-rank test.

To further investigate the mechanisms responsible for survival in our model, we compared the data from the animals that did not survive the repair phase with those that did (Fig. 5). As sampling times were different between survivors and nonsurvivors, we first split the nonsurvivor group using the median ventilation time. There were no differences in any of the measured molecules between these two subgroups, thus discarding an influence of the temporal difference of sample collection (data not shown).

Regarding indexes of lung injury, survivors showed a more severe degree of histological injury (Fig. 5B, P = 0.05). The total number of pneumocytes and the percentage of Ki-67-positive nuclei were also significantly higher in survivors (Fig. 5, C and D, P = 0.02 and P = 0.03, respectively). Despite the absence of differences in the wet-to-dry ratio (Fig. 5G, P = 0.32), survivors showed a decreased protein content in BALF (Fig. 5H, P = 0.05). The leukocyte counts in BALF were

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**Fig. 3. Gelatinases during injury and repair.** Matrix metalloproteinases (MMP)-9 (A and C) and -2 (B and D) were measured in lung tissue and BALF. Mechanical ventilation induced significant increases in both enzymes, which kept elevated levels during repair. Values are means ± SE. E and F: representative gelatin zymographies (lung tissue and BALF, respectively). #P < 0.05 in post hoc test compared with baseline. †P < 0.05 in post hoc test compared with injury.
higher in survivors (2.3 ± 0.3 vs. 0.8 ± 0.2 × 10^3 cells/ml, P = 0.01), with higher neutrophil and macrophage counts (Fig. 5I, P = 0.04 and P = 0.02, respectively). The number of MPO-positive cells was also higher in survivors (Figs. 4 and 5I). The differences in immune mediators are presented in Table 2. There were no differences in TNF-α, IFN-γ or LIX; however, lower IL-4 levels and a trend toward higher IL-10 levels were observed in survivors. Nevertheless, the most striking difference was the eightfold increase of MIP-2 levels in survivors. Finally, extracellular matrix proteins were compared (Fig. 5, K–M). Lower collagen levels were found in survivors (P = 0.05). MMP-2 doubled its activity in the surviving mice (P < 0.05), with no significant differences in MMP-9 (P = 0.531). These results suggest that an adequate inflammatory response and tissue remodeling are both important phenomena in lung repair.

**MMP-2 promotes alveolar wound healing in murine and human cells.** The in vivo experiments revealed some mediators that could be responsible for the direct induction of injury or repair. Then we tested the effects of these molecules on wound healing using cell culture models. Control cultures (without BALF) showed a slower closure rate than those cultured with BALF, suggesting that cell growth is promoted by factors present in BALFs and not secreted by cultured cells. TNF-α, IL-4, or MIP-2 inhibition had no effect on the wound closure rate (data not shown). However, MMP-2 inhibition in cultures supplemented with BALF from mice during repair delayed wound closure after 12 h (Fig. 6A, P = 0.04 in a repeated-measurements ANOVA). Likewise, MMP-2 inhibition in a similar model using human alveolar A549 cells and BALF from ARDS patients impaired wound healing (Fig. 6B, P = 0.007). Pan-MMP inhibition with doxycycline further impaired wound healing in both murine and human culture models, but also induced changes in cell morphology and adhesion, rendering closure measurements unreliable. These results illustrate the relevance of MMPs in the healing process and, specifically, confirm that MMP-2 promotes epithelial cell migration and suggests that it contributes to alveolar repair after VILI or ARDS.

**MMP inhibition impairs lung repair.** The effects of MMPs on lung repair were studied using an in vivo model. The mortality of doxycycline-treated mice during the repair phase was 75%. Compared with vehicle-treated mice with the same ventilatory time, MMP inhibition results in higher histological scores of lung injury (Fig. 7). These results confirm that MMPs may be beneficial during lung repair after VILI.

**DISCUSSION**

Our results demonstrate that, in the present experimental models, repair after VILI can be rapidly achieved. Additionally, this study demonstrates the key role of extracellular matrix processing during lung repair, as lower levels of lung collagen and higher levels of MMP-2 were found in mice surviving lung injury. Finally, we have shown that MMP-2 promotes epithelial repair after VILI and in patients with early...
ARDS. Although all of these mechanisms have been described in other forms of injury and repair, this work shows that inflammation, matrix remodeling, and cell migration act in an orchestrated fashion to repair the injured lung.

Inflammation and lung repair. Nin et al. (28) demonstrated that, after high-volume ventilation, lung injury could be reversed with restoration of spontaneous breathing. Our results confirm these findings, showing a partial repair after 4 hours of noninjurious ventilation. The onset of this repair phase is characterized by the reversal of different pathogenetic mechanisms of VILI, namely lung edema, inflammatory response, and tissue remodeling. One of the most striking changes was the normalization of the alveolocapillary permeability, demonstrated by the rapid decrease in the protein content of the BALF. The reabsorption of lung edema and the normalization of fluid and protein filtration are key steps in recovering from ARDS (39).

Table 2. Differences in immune mediators in lung tissue between survivors and nonsurvivors to the repair phase of the experiment

<table>
<thead>
<tr>
<th></th>
<th>Survivors</th>
<th>Nonsurvivors</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>4.92 ± 1.29</td>
<td>7.08 ± 1.86</td>
<td>0.370</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.84 ± 1.86</td>
<td>4.44 ± 1.32</td>
<td>0.359</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.59 ± 0.18</td>
<td>3.12 ± 0.51</td>
<td>0.011</td>
</tr>
<tr>
<td>IL-10</td>
<td>26.43 ± 5.07</td>
<td>16.62 ± 1.05</td>
<td>0.105</td>
</tr>
<tr>
<td>MIP-2</td>
<td>721.41 ± 196.5</td>
<td>135.56 ± 13.44</td>
<td>0.024</td>
</tr>
<tr>
<td>LIX</td>
<td>432.51 ± 96.1</td>
<td>587.79 ± 161.37</td>
<td>0.400</td>
</tr>
</tbody>
</table>

Values are means ± SE in pg/mg protein. MIP-2, macrophage inflammatory protein-2; LIX, LPS-induced CXC chemokine.

Inflammation and lung repair. Nin et al. (28) demonstrated that, after high-volume ventilation, lung injury could be reversed with restoration of spontaneous breathing. Our results confirm these findings, showing a partial repair after 4 hours of noninjurious ventilation. The onset of this repair phase is characterized by the reversal of different pathogenetic mechanisms of VILI, namely lung edema, inflammatory response, and tissue remodeling. One of the most striking changes was the normalization of the alveolocapillary permeability, demonstrated by the rapid decrease in the protein content of the BALF. The reabsorption of lung edema and the normalization of fluid and protein filtration are key steps in recovering from ARDS (39).

Regarding inflammation, high-pressure ventilation triggers a mechanotransduction process (26) that leads to changes in cell populations and immune mediators. The trend to lower absolute cell counts during injury has been explained by the dilution
Our study supports the existence of some of these phenomena directly releasing defensins and other growth factors that differentiate inflammation toward an anti-inflammatory response; and by modulating both extracellular matrix degradation and epithelial cell migration through different mechanisms (24): release of proteases (MMP-2), a decrease of collagen levels, and the restoration of IL-10, an anti-inflammatory cytokine with beneficial effects in VILI (2, 20).

The elevation of proinflammatory cytokines, such as TNF-α or IFN-γ, and a decrease in anti-inflammatory mediators (IL-10) is in keeping with previous results (31). Restoration of low pressures induced a rapid normalization of these cell populations and cytokines. We cannot discard that this decrease is part of the normal cytokine kinetics during the inflammatory response. However, other studies (21) with prolonged injurious ventilation found elevated levels of TNF-α at later time points (4 h). None of these mediators had a significant impact in the wound-healing model. Therefore, these molecules could be involved in the changes in permeability or in the systemic response to VILI (1), but not directly in epithelial repair.

The increased cell counts seen during repair can be driven by the release of chemokines, such as MIP-2. This is especially evident in mice surviving the repair phase, which show higher levels of MIP-2 and inflammatory cells. This inflammatory infiltrate is, in part responsible for the higher histological scores seen in this group. Previous results have demonstrated that both MIP-2 (6) and inflammatory cell infiltration (15) can promote VILI. The findings presented here suggest that this response also drives lung repair. These dual effects of inflammation during VILI could explain the good responses seen with anti-inflammatory drugs, such as steroids, in experimental models of VILI (29) and the lack of benefits seen in the clinical practice (38).

**MMP-2 in lung repair.** Our laboratory has shown that MMP-2 plays a relevant role in lung repair after VILI. This gelatinase is constitutively secreted from a great variety of cells, including the lung epithelium, and in response to stretch (19). There is abundant evidence that MMP-2 is essential for cell migration, in both normal and cancer cells, and that it can also promote the cleavage of interstitial proteins (24). This is in agreement with our data, which show decreased levels of collagen and increased MMP-2 in survivors after lung injury, and is also consistent with the impairment of epithelial cell migration after MMP-2 inhibition. The involvement of this enzyme in other models of lung injury and repair has been described previously (7). Furthermore, MMP-2 can also facilitate the resolution of inflammation by cleavage of immune mediators (17), although the relevance of this mechanism has not been studied in the context of acute lung injury or VILI.

We have also described a role of MMP-2 in human ARDS. An increase in different proteases in the BALF from patients with this syndrome has been described (36). Our results show during the repair phase after VILI, including the release of proteases (MMP-2), a decrease of collagen levels, and the restoration of IL-10, an anti-inflammatory cytokine with beneficial effects in VILI (2, 20).

Both neutrophils (18) and macrophages (35) are involved in wound healing. In skin wounds, the extracellular matrix builds a scar that replaces normal epithelium. However, this repair mechanism within the lungs results in fibrosis and impairs gas exchange and respiratory mechanics. In this setting, reepithelialization and collagen degradation are essential, as shown in other models of tissue repair (18). Inflammatory cells can modulate both extracellular matrix degradation and epithelial cell migration through different mechanisms (24): release of proteases that cleave collagen, gelatin, and elastin; modulation of inflammation toward an anti-inflammatory response; and by directly releasing defensins and other growth factors that directly stimulate epithelial cell migration and proliferation (27). Our study supports the existence of some of these phenomena caused by the increase in edema and attachment of cells to the alveolar epithelium (22). The specific decrease in macrophages has also been described (15). Although it has been recently reported that lymphocytes have a role in lung repair (10), we did not find any significant differences in our model.

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that human alveolar cells cultured in the presence of this BALF show a delay in wound closure when MMP-2 is inhibited, supporting the role of this protease in repair. O’Kane et al. have shown similar results involving MMP-9 in ARDS patients (30), which also resembles the results from a VILI model using Mmp9 knockout mice (3). As both are gelatinases, it is probable overlapping effects take place. Furthermore, nonspecific MMP inhibition resulted in further impairment of in vitro wound healing. Although these findings are limited by the lack of data using cell stretch models, they suggest that different MMPs, including gelatinases, induce alveolar repair by promoting epithelial migration. Moreover, the results showing impaired repair after VILI in doxycycline-treated mice confirm this result. Therefore, preventive MMP inhibition can protect against VILI (14), but blocking these enzymes during the repair phase may be detrimental. These opposite effects illustrate the relationships between tissue remodeling, initial damage, and subsequent repair, and highlight the critical importance of timing when targeting these processes.

According to our results, overexpression of these enzymes could be of interest in the context of lung injury and mechanical ventilation. There are different drugs that promote MMP expression. Salbutamol increases MMP-9 expression in neutrophils and facilitates lung edema reabsorption (30). MMP-2 can be secreted in response to transforming growth factor-β (25) [which is released during lung injury (34)] or nitric oxide (8), but their therapeutic use on lung repair is yet to be studied.

Finally, the decreased closure rate seen in cell cultures without BALF suggests that there are other factors that may promote healing, which have not been identified in this study. Knowledge of molecules involved in epithelial repair, which may include growth factors, could offer new therapeutic approaches to lung injury.

Conclusions. In summary, we have shown that VILI is potentially reversible, and that the repair process depends on both an adequate inflammatory response and extracellular matrix remodeling. Regarding the latter, we have found that MMPs, and specifically MMP-2, promote lung repair by facilitating epithelial cell migration. This repair mechanism is also relevant in human ARDS. The finding of molecules directly involved in tissue repair constitutes a new therapeutic opportunity for the treatment of lung injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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